

**Table 1. Susceptibility of mice to intracerebral DEN4 infection is age-dependent<sup>a</sup>**

Virus	Mean virus titer ( $\log_{10}$ PFU/g brain) $\pm$ SE following inoculation at indicated age (days)		
	7	14	21
2A-13	>6.0	4.0 $\pm$ 0.2	3.1 $\pm$ 0.2
rDEN4	>6.0	3.3 $\pm$ 0.4	3.3 $\pm$ 0.2
rDEN4 $\Delta$ 30	>6.0	3.6 $\pm$ 0.2	2.8 $\pm$ 0.3

<sup>a</sup> Groups of 4 or 5 Swiss Webster mice were inoculated intracerebrally with  $10^5$  PFU virus in a 30  $\mu$ l inoculum. After 5 days, brains were removed, homogenized and titered in Vero cells. SE = Standard error.

**TABLE 2** Temperature-sensitive (*ts*) and mouse brain attenuation (*att*) phenotypes of 5-FU mutant DEN4 viruses.

Phenotype	Virus	Mean virus titer (log <sub>10</sub> PFU/ml) at indicated temp. (°C)										Virus replication in suckling mice <sup>b</sup>		
		Vero cells					HuH-7 cells					n	Mean titer ± SE (log <sub>10</sub> PFU/g brain)	Mean log <sub>10</sub> reduction from wt <sup>d</sup>
		35	37	38	39	Δ <sup>a</sup>	35	37	38	39	Δ			
<i>ts</i> in Vero and HuH-7 cells	wt (not <i>ts</i> )	7.8	7.7	7.6	7.3	0.5	7.8	7.7	7.4	6.4	1.4	66	6.6 ± 0.1c	-
	rDEN4	6.5	6.4	6.4	6.0	0.5	7.1	6.7	6.0	5.5	1.6	66	6.1 ± 0.1c	-
	rDEN4Δ30	6.3	6.1	6.1	5.7	0.6	6.9	6.3	5.9	4.7	2.2	64	5.6 ± 0.1c	0.5
	695	6.2	6.0	5.2	2.6 <sup>e</sup>	3.6	6.5	5.5	3.8	<1.6	>4.9	6	3.0 ± 0.2	3.2
	816	6.8	6.4	5.8	3.9	2.9	7.5	6.2	5.5	3.1	4.4	6	3.3 ± 0.4	2.9
	773	7.4	6.6	6.0	3.1	4.3	7.7	6.1	5.2	3.1	4.6	12	3.7 ± 0.1	2.6
	489	7.3	6.6	6.1	3.3	4.0	7.3	6.7	5.4	3.0	4.3	6	4.5 ± 0.5	2.3
	173	7.0	6.1	3.2	2.9	4.1	7.0	3.2	3.0	2.1	4.9	18	4.7 ± 0.4	2.2
	509	6.2	5.8	5.5	3.4	2.8	6.5	6.1	4.5	<1.6	>4.9	6	4.9 ± 0.3	1.9
	938	7.1	6.5	5.6	3.1	4.0	7.2	6.4	5.6	3.1	4.1	6	5.1 ± 0.2	1.7
<i>ts</i> in only HuH-7 cells	1033	6.7	6.0	5.9	4.1	2.6	6.9	5.6	4.7	<1.6	>5.3	12	4.7 ± 0.2	1.7
	239	7.6	6.8	5.6	3.3	4.3	7.6	6.7	4.7	2.5	5.1	12	4.7 ± 0.3	1.5
	793	6.5	5.8	5.3	4.0	2.5	7.2	6.8	5.6	<1.6	>5.6	6	5.4 ± 0.3	1.4
	759	7.2	6.9	6.4	4.7	2.5	7.5	6.8	6.3	3.1	4.4	12	5.1 ± 0.1	1.4
	718	6.1	5.9	5.3	3.5	2.6	7.0	6.5	5.7	1.7	5.3	12	5.0 ± 0.3	1.4
	473	6.7	6.3	5.4	2.0	4.7	7.2	6.7	3.7	1.9	5.3	12	5.1 ± 0.3	1.2
	686	7.0	6.7	6.7	6.4	0.6	7.3	6.8	6.4	2.2	5.1	12	2.7 ± 0.2	3.8
	967	6.8	6.4	6.4	5.1	1.7	6.8	6.4	5.4	<1.6	>5.2	6	3.6 ± 0.2	2.9
	992	7.3	7.1	6.8	5.9	1.4	7.4	6.9	5.0	<1.6	>5.8	6	3.8 ± 0.1	2.7
	571	6.9	7.0	6.4	4.6	2.3	7.0	6.3	5.2	<1.6	>5.4	6	4.4 ± 0.4	2.4
<i>ts</i> in only HuH-7 cells	605	7.6	7.5	7.1	6.9	0.7	7.8	7.2	6.8	<1.6	>6.2	12	4.5 ± 0.4	2.1
	631	7.1	6.9	6.8	5.0	2.1	7.3	7.1	6.5	<1.6	>5.7	12	4.8 ± 0.3	1.9
	1175	7.4	7.1	6.9	5.3	2.1	7.6	6.5	4.7	3.3	4.3	12	4.7 ± 0.2	1.7

- <sup>a</sup> Reduction in titer ( $\log_{10}$  PFU/ml) at 39°C compared to titer at permissive temperature (35°C).
- <sup>b</sup> Groups of 6 suckling mice were inoculated i.c. with  $10^4$  PFU virus in a 30  $\mu$ l inoculum. Brains were removed 5 days later, homogenized, and titered in Vero cells.
- <sup>c</sup> Average of 11 experiments with a total of 64 to 66 mice per group.
- <sup>d</sup> Determined by comparing mean viral titers of mice inoculated with mutant virus and the 2A-13 wt control in the same experiment (n= 6 or 12).
- <sup>e</sup> Underlined values indicate a 2.5 or 3.5  $\log_{10}$  PFU/ml reduction in titer in Vero cells or HuH-7 cells, respectively, at indicated temp when compared to titer at permissive temp (35°C).

**Table 3.** Nucleotide and amino acid differences of the 5-FU mutant viruses which are *ts* in both Vero and HuH-7 cells.

Virus	Mutations in UTR or coding region that result in an amino acid substitution				Mutations in coding region that do not result in an amino acid substitution		
	Nucleotide position	Gene/region	Nucleotide change	Amino Acid change <sup>b</sup>	Nucleotide position	Gene	Nucleotide change
173 <sup>a</sup>	7163	NS4B	A > C	L2354F	10217	NS5	A > U
	7849	NS5	A > U	N2583I			
	8872	NS5	A > G	K2924R			
239 <sup>a</sup>	4995	NS3	U > C	S1632P	7511	NS4B	G > A
					10070	NS5	U > C
473 <sup>a</sup>	4480	NS2B	U > C	V1460A	7589	NS5	G > A
	4995	NS3	U > C	S1632P	10070	NS5	U > C
489 <sup>a</sup>	4995	NS3	U > C	S1632P	2232	E	U > C
					3737	NS2A	C > U
509 <sup>a</sup>	4266	NS2B	A > G	S1389G	none		
	8092	NS5	A > G	E2664G			
695	40	5' UTR	U > C	n/a	1391	E	A > G
	1455	E	G > U	V452F			
	6106	NS3	A > G	E2002G			
	7546	NS4B	C > U	A2482V			
718	2280	E	U > C	F727L	none		
	4059	NS2A	A > G	I1320V			
	4995	NS3	U > C	S1632P			
	7630	NS5	A > G	K2510R			
	8281	NS5	U > C	L2727S			
759 <sup>a</sup>	4995	NS3	U > C	S1632P	none		
	8020	NS5	A > U	N2640I			
773 <sup>a</sup>	4995	NS3	U > C	S1632P	none		
793	1776	E	G > A	A559T	5771	NS3	U > C
	2596	NS1	G > A	R832K	7793	NS5	U > A
	2677	NS1	A > G	D859G			
	4387	NS2B	C > U	S1429F			
816 <sup>a</sup>	4995	NS3	U > C	S1632P	6632	NS4A	G > A
	7174	NS4B	C > U	A2358V	6695	NS4A	G > A

Virus	Mutations in UTR or coding region that result in an amino acid substitution				Mutations in coding region that do not result in an amino acid substitution		
	Nucleotide position	Gene/region	Nucleotide change	Amino Acid change <sup>b</sup>	Nucleotide position	Gene	Nucleotide change
938 <sup>a</sup>	3442	NS1	A > G	E1114G	747	prM	U > C
	4995	NS3	U > C	S1632P	4196	NS2b	U > C
	10275	3' UTR	A > U	n/a	6155	NS3	G > A
1033 <sup>a</sup>	4907	NS3	A > U	L1602F	548	prM	C > U
	8730	NS5	A > C	N2877H			
	9977	NS5	G > A	M3292I			

<sup>a</sup> Viruses that contain mutation(s) resulting in an a.a. substitution in only a NS gene(s) and/or nucleotide substitutions in the UTRs are indicated; i.e. no a.a. substitutions are present in the structural proteins (C-prM-E).

<sup>b</sup> Amino acid position in DEN4 polyprotein beginning with the methionine residue of the C protein (nt 102-104) as residue #1. Wild-type amino acid on left of amino acid position; mutant amino acid on right.

**Table 4.** Nucleotide and amino acid differences of the 5-FU mutant viruses which are *ts* in only HuH-7 cells.

Virus	Mutations in UTR or coding region that result in an amino acid substitution				Mutations in coding region that do not result in an amino acid substitution		
	Nucleotide position	Gene/region	Nucleotide change	Amino acid change <sup>b</sup>	Nucleotide position	Gene	Nucleotide change
571	586	prM	U > C	V162A	6413	NS4A	U > C
	7163	NS4B	A > U	L2354F			
	7947	NS5	G > A	G2616R			
605	1455	E	G > U	V452F	none		
	7546	NS4B	C > U	A2482V			
631	595	prM	A > G	K165R	1175	E	G > A
	6259	NS3	U > C	V2053A	5174	NS3	A > G
	7546	NS4B	C > U	A2482V			
686 <sup>a</sup>	3575	NS2A	G > A	M1158I	4604	NS3	A > G
	4062	NS2A	A > G	T1321A	7937	NS5	A > G
	7163	NS4B	A > U	L2354F			
967	2094	E	G > C	A665P	4616	NS3	C > U
	2416	E	U > C	V772A			
	7162	NS4B	U > C	L2354S			
	7881	NS5	G > A	G2594S			
992 <sup>a</sup>	5695	NS3	A > G	D1865G	3542	NS2A	A > G
	7162	NS4B	U > C	L2354S			
1175 <sup>a</sup>	7153	NS4B	U > C	V2351A	6167	NS3	U > C
	10186	NS5	U > C	I3362T	10184	NS5	G > A
	10275	3' UTR	A > U	n/a			

<sup>a</sup> Viruses that contain mutation(s) resulting in an a.a. substitution in only a NS gene(s) and/or nucleotide substitutions in the UTRs are indicated; i.e. no a.a. substitutions are present in the structural proteins.

<sup>b</sup> Amino acid position in DEN4 polyprotein beginning with the methionine residue of the C protein (nt 102-104) as residue #1. Wild-type amino acid on left of amino acid position; mutant amino acid on right.

**TABLE 5.** Mutations which are represented in multiple 5-FU mutant DEN4 viruses.

Nucleotide position	Gene/region	Nucleotide change	Amino acid change	Number of viruses with "sister" mutations
1455	E	G > U	val > phe	2
4995	NS3	U > C	ser > pro	8
7162	NS4B	U > C	leu > ser	2
7163	NS4B	A > U or C	leu > phe	3
7546	NS4B	C > U	ala > val	3
10275	3' UTR	A > U	n/a <sup>a</sup>	2

<sup>a</sup> not applicable

**Table 6.** Addition of *ts* mutation 4995 to rDEN4Δ30 confers a *ts* phenotype and further attenuates its replication in suckling mouse brain.

Virus	Mean virus titer (log <sub>10</sub> PFU/ml) at indicated temp (°C)										Replication in suckling mice <sup>b</sup>	
	Vero cells					HuH-7 cells					Mean virus titer ± SE (log <sub>10</sub> PFU/g brain)	Mean log <sub>10</sub> reduction from w <sup>f</sup>
	35	37	38	39	Δ <sup>a</sup>	35	37	38	39	Δ		
2A-13	7.1	7.1	6.9	6.8	0.3	7.4	7.3	6.7	6.4	1.0	6.5 ± 0.1	-
rDEN4	7.0	6.8	6.6	6.4	0.6	7.5	7.3	6.7	6.4	1.1	6.1 ± 0.2	-
rDEN4Δ30	7.0	6.7	6.2	6.2	0.8	7.5	7.0	6.5	5.1	2.4	5.9 ± 0.1	0.2
rDEN4-4995	5.7	4.9	3.6	<u>≤1.6</u>	>4.1	6.4	5.7	4.0	<u>≤1.6</u>	>4.8	3.2 ± 0.2	2.9
rDEN4Δ30-4995	5.9	4.9	3.9	<u>≤1.6<sup>d</sup></u>	>4.3	6.4	5.6	4.4	<u>≤1.6</u>	>4.8	3.0 ± 0.3	3.1

<sup>a</sup> Reduction in titer (log<sub>10</sub>PFU/ml) at 39°C compared to titer at permissive temperature (35°C).

<sup>b</sup> Groups of 6 suckling mice were inoculated i.c. with 10<sup>4</sup> PFU virus in a 30 μl inoculum. Brains were removed 5 days later, homogenized, and titered in Vero cells. The limit of detection is 2.0 log<sub>10</sub>PFU/g brain.

<sup>c</sup> Determined by comparing mean viral titers of mice inoculated with sample virus and rDEN4 control.

<sup>d</sup> Underlined values indicate a 2.5 or 3.5 log<sub>10</sub>PFU/ml reduction in titer in Vero cells or HuH-7 cells, respectively, at indicated temperature when compared to permissive temperature.



**Table 7. Temperature-sensitive (*ts*) and mouse brain attenuation (*att*) phenotypes of 5-FU DEN4 mutant viruses which exhibit a small plaque (*sp*) phenotype.**

Phenotype				Virus	Mean virus titer (log <sub>10</sub> PFU/ml) at indicated temp (°C)										Replication in suckling mice <sup>b</sup>				
sp		ts			Vero		HuH-7		Mean virus titer										Mean log <sub>10</sub> reduction from wt <sup>d</sup>
									(log <sub>10</sub> PFU/g brain)										
Vero		HuH-7	Vero	HuH-7	35	37	38	39	Δ <sup>a</sup>	35	37	38	39	Δ	n	Mean virus titer (log <sub>10</sub> PFU/g brain)	Mean log <sub>10</sub> reduction from wt <sup>d</sup>		
—	—	—	—	—	7.9	7.5	7.7	7.2	0.7	7.9	7.7	7.3	6.9	1.0	66	6.6 ± 0.1 <sup>c</sup>	—		
—	—	—	—	2A-13	7.9	7.6	7.7	7.3	0.6	8.1	7.6	7.5	6.7	1.4	66	6.1 ± 0.1 <sup>c</sup>	—		
—	—	—	—	rDEN4	7.3	6.6	6.6	6.1	1.2	7.3	7.2	6.9	5.9	1.4	64	5.6 ± 0.1 <sup>c</sup>	0.5		
+	+	+	+	rDEN4Δ30	6.6 <sup>x</sup>	5.5	3.8	<1.6 <sup>c</sup>	≥5.0	6.6 <sup>x</sup>	4.9	5.0	<1.6	≥5.0	6	2.1 ± 0.1	5.1		
+	+	+	+	574	5.3 <sup>x</sup>	4.8	3.9	<1.6	≥3.7	4.0 <sup>x</sup>	2.4	2.0	<1.6	≥2.4	6	2.7 ± 0.2	4.1		
+	+	+	+	1,269	6.3 <sup>x</sup>	5.2	4.5	3.8	2.5	5.5 <sup>x</sup>	3.7	2.3	<1.6	≥3.9	12	3.2 ± 0.4	3.7		
+	+	+	+	1,189	5.8 <sup>x</sup>	5.6	5.6	3.7	2.1	6.2 <sup>x</sup>	6.0	5.7	5.0	1.2	12	1.9 ± 0.1	4.6		
+	+	—	—	569	5.0 <sup>x</sup>	4.7	4.2	2.7	2.3	5.6 <sup>x</sup>	5.3	4.5	2.6	3.0	12	2.0 ± 0.1	4.2		
+	+	—	—	761	7.0	6.8	5.6	2.6	4.4	6.7 <sup>x</sup>	4.3	<1.6	2.0	4.7	6	2.2 ± 0.1	4.7		
—	+	+	+	506	5.1	4.2	2.6	<1.6	≥3.5	5.7 <sup>x</sup>	3.0	3.0	<1.6	≥4.1	6	2.9 ± 0.3	4.5		
—	+	+	+	1,136	6.9	5.8	5.8	2.9	4.0	7.0 <sup>x</sup>	5.8	5.2	2.5	4.5	6	2.2 ± 0.1	4.2		
—	+	+	+	1,029	6.9	5.8	4.7	3.9	3.0	5.8 <sup>x</sup>	4.1	3.3	1.9	3.9	12	2.6 ± 0.2	3.9		
—	+	+	+	1,081	6.9	6.5	5.9	4.0	2.9	7.1 <sup>x</sup>	5.3	4.4	<1.6	≥5.5	6	3.1 ± 0.7	3.8		
—	+	+	+	529	6.7	6.4	6.2	2.5	4.2	5.7 <sup>x</sup>	3.0	2.9	1.9	3.8	6	2.7 ± 0.3	3.7		
—	+	+	+	1,114	7.3	7.2	6.8	3.8	3.5	7.4 <sup>x</sup>	5.3	4.1	3.0	4.4	12	3.5 ± 0.1	2.9		
—	+	+	+	922	6.9	5.9	4.3	1.5	5.4	7.1 <sup>x</sup>	5.4	3.6	<1.6	≥5.5	12	6.1 ± 0.3	0.9		
—	+	+	+	311	6.6	5.7	4.5	3.1	3.5	7.0 <sup>x</sup>	5.5	4.1	2.0	5.0	6	6.0 ± 0.1	0.9		
—	+	+	+	326	7.1	6.8	6.8	6.1	1.0	7.2 <sup>x</sup>	6.4	5.8	2.8	4.4	6	2.2 ± 0.1	4.7		
—	+	—	+	1,104	7.1	7.0	6.7	5.6	1.5	7.3 <sup>x</sup>	6.3	5.6	3.0	4.3	6	2.4 ± 0.3	4.5		
—	+	—	+	952	6.5	6.0	5.9	5.7	0.8	6.9 <sup>x</sup>	6.1	5.0	3.1	3.8	12	4.4 ± 0.4	2.3		
—	+	—	+	738	7.4	7.3	7.4	5.8	1.6	7.4 <sup>x</sup>	6.6	4.5	<1.6	≥5.8	12	4.5 ± 0.4	2.0		
—	+	—	+	1,083	7.5	7.1	6.9	5.5	2.0	7.5 <sup>x</sup>	6.6	5.6	4.8	2.7	6	2.9 ± 0.2	3.5		
—	+	—	—	1,096	7.0	6.9	6.6	6.3	0.7	6.9 <sup>x</sup>	5.7	4.4	4.0	2.9	6	3.9 ± 0.6	2.6		
—	+	—	—	1,021															

—	+	—	1,023	6.6	6.4	6.0	5.8	0.8	6.1*	5.6	4.7	3.3	2.8	12	4.2 ± 0.3	2.3
—	+	—	1,012	7.5	7.1	7.0	5.7	1.8	7.4*	6.8	6.8	5.6	1.8	6	6.1 ± 0.1	0.8

- \* Reduction in mean virus titer ( $\log_{10}$ PFU/ml) at 39°C compared to permissive temperature (35°C).
- <sup>b</sup> Groups of 6 suckling mice were inoculated i.c. with  $10^4$  PFU virus. Brains were removed 5 days later, homogenized, and titered in Vero cells.
- <sup>c</sup> Average of 11 experiments with a total of 64 to 66 mice per group.
- <sup>d</sup> Determined by comparing mean viral titers of mice inoculated with mutant virus and concurrent 2A-13 wild type (*wt*) virus control ( $n = 6$  or 12).
- <sup>e</sup> Underlined values indicate a 2.5 or 3.5  $\log_{10}$ PFU/ml reduction in titer in Vero cells or HuH-7 cells, respectively, at indicated temperature when compared to permissive temperature (35°C).
- <sup>x</sup> Small plaque size at 35°C; small plaques have a diameter of <1.0 mm compared to wild type plaque diameter of 1.5 - 2.0 mm in Vero cells, or a diameter of <0.4 mm compared to wild type plaque diameter of 0.75 to 1.0 mm in HuH-7 cells.

**Table 8.** Viruses with both *ts* and *sp* phenotypes are more restricted in replication in mouse brain than those with only a *ts* phenotype.

Cell culture phenotype	Number of viruses	Mean log <sub>10</sub> reduction in virus titer from control <sup>b, c</sup>
<i>ts</i> <sup>a</sup>	20	2.1 ± 0.2
<i>sp</i>	6	3.0 ± 0.6
<i>ts</i> / <i>sp</i>	16	3.5 ± 0.3

<sup>a</sup> 20 *ts* mutant viruses without an *sp* phenotype were previously described (Example 1).

<sup>b</sup> Determined by comparing mean viral titers of groups of mice inoculated with mutant virus and concurrent 2A-13 parallel-passaged control virus.

<sup>c</sup> Significant difference between *ts* group and *ts* / *sp* group, Tukey-Kramer test (P < 0.05)

**Table 9.** Nucleotide and amino acid differences of the 5-FU mutant DEN4 viruses which produce small plaques in both Vero and HuH-7 cells.

Virus	Mutations in UTR or in coding regions that result in an amino acid substitution				Mutations in coding regions that do not result in an amino acid substitution		
	Nucleotide position	Gene/region	Nucleotide change	Amino acid change <sup>b</sup>	Nucleotide position	Gene	Nucleotide change
569	826	prM	G > A	R242K	1946	E	C > U
	832	prM	C > U	P244L			
	7546	NS4B	C > U	A2482V			
	10275	3' UTR	A > U	n/a			
	10279	3' UTR	A > U	n/a			
574	1455	E	G > U	V452F	1349	E	C > U
	1963	E	U > C	V621A			
	3880	NS2A	A > G	K1260R			
	7546	NS4B	C > U	A2482V			
	7615	NS5	A > G	N2505S			
	10413	3' UTR	A > G	n/a			
761	424	C	U > C	I108T	none		
	2280	E	U > C	F727L			
	7131	NS4B	A > G	T2344A			
	7486	NS4B	A > G	N2462S			
1189a	3303	NS1	A > G	R1068G	6719	NS4A	U > C
	4812	NS3	G > A	V1571I			
	5097	NS3	G > A	D1666N			
	7182	NS4B	G > A	G2361S			
1269	2112	E	U > C	F671L	542	prM	C > U
	3256	NS1	G > A	G1052E			
	3993	NS2A	U > C	F1298L			
	7183	NS4B	G > U	G2361V			

<sup>a</sup> Virus contains missense mutations in only the non-structural genes.

<sup>b</sup> Amino acid position in DEN4 polyprotein beginning with the methionine residue of the C protein (nt 102-104).

Wild type amino acid on left of amino acid position; mutant amino acid on right.

**Table 10.** Nucleotide and amino acid differences of the 5-FU mutant DEN4 viruses which produce small plaques in only HuH-7 cells.

Virus	Mutations in UTR or in coding regions that result in an amino acid substitution				Mutations in coding regions that do not result in an amino acid substitution		
	Nucleotide position	Gene/region	Nucleotide change	Amino acid change <sup>b</sup>	Nucleotide position	Gene	Nucleotide change
311	1519	E	A > G	N473S	6761	NS4A	C > U
	2305	E	G > A	R735K	10070	NS5	U > C
	4896	NS3	G > U	A1599S			
326	1587	E	C > U	P496S	1523	E	G > A
	7546	NS4B	C > U	A2482V	6080	NS3	U > C
					10070	NS5	U > C
506	1455	E	G > U	V452F	3887	NS2A	A > G
	1902	E	G > A	V601M	5789	NS3	G > C
	7546	NS4B	C > U	A2482V			
	10275	3' UTR	A > U	n/a			
529	777	prM	U > C	S226P	none		
	4641	NS3	A > G	I1514V			
	7153	NS4B	U > C	V2351A			
	8245	NS5	U > C	I2715T			
	10279	3' UTR	A > C	n/a			
738 <sup>a</sup>	3540	NS2A	G > A	E1147K	none		
	7162	NS4B	U > C	L2354S			
922 <sup>a</sup>	4306	NS2B	A > G	N1402S	7736	NS5	G > A
	5872	NS3	C > U	T1924I			
	7163	NS4B	A > U	L2354F			
	10279	3' UTR	A > C	n/a			
952	1449	E	G > U	V450L	none		
	1455	E	G > U	V452F			
	7546	NS4B	C > U	A2482V			
	7957	NS5	U > C	V2619A			
	9543	NS5	A > G	I3148V			
1012	1542	E	A > G	K481E	953	E	A > G
	7162	NS4B	U > C	L2354S	1205	E	G > A
	10542	3' UTR	A > G	n/a	4425	NS2B	U > C
1021	2314	E	U > C	I738T	665	prM	C > A
	3205	NS1	C > U	A1035V	5750	NS3	C > U
	4029	NS2A	U > C	C1310R	9959	NS5	C > U
	7163	NS4B	A > C	L2354F			

Virus	Mutations in UTR or in coding regions that result in an amino acid substitution				Mutations in coding regions that do not result in an amino acid substitution		
	Nucleotide position	Gene/region	Nucleotide change	Amino acid change <sup>b</sup>	Nucleotide position	Gene	Nucleotide change
	10275	3' UTR	A > U	n/a			
	10279	3' UTR	A > U	n/a			
1023	2283	E	G > A	G728R	1001	E	C > U
	7182	NS4B	G > A	G2361S	1958	E	A > G
					3873	NS2a	U > C
					8486	NS5	C > U
1029	850	prM	C > U	A250V	3867	NS2a	C > U
	3087	NS1	A > G	T996A			
	4891	NS3	U > C	I1597T			
1081 <sup>a</sup>	2650	NS1	A > G	N850S	6326	NS3	C > U
	7163	NS4B	A > U	L2354F	9146	NS5	C > U
1083 <sup>a</sup>	3702	NS2A	G > A	A1201T	3353	NS1	A > G
	7153	NS4B	U > C	V2351A	6155	NS3	G > A
	10634	3' UTR	U > C	n/a			
1096	892	prM	G > A	R264Q	665	prM	C > A
	7163	NS4B	A > C	L2354F	4427	NS2b	G > A
	8659	NS5	C > U	P2853L			
1104	1692	E	G > A	V531M	none		
	5779	NS3	C > U	A1893V			
	7546	NS4B	C > U	A2482V			
1114	709	prM	A > G	K203R	1076	E	U > C
	3693	NS2A	A > G	I1198V	1182	E	C > U
	4614	NS3	U > C	F1505L	5690	NS3	C > U
	7546	NS4B	C > U	A2482V			
	9942	NS5	A > G	T3281A			
1136 <sup>a</sup>	3771	NS2A	A > G	R1224G	5621	NS3	A > G
	4891	NS3	U > C	I1597T			
	10275	3' UTR	A > U	n/a			

<sup>a</sup> Viruses that contain missense mutations in only the non-structural genes and/or mutations in the UTRs.

<sup>b</sup> Amino acid position in DEN4 polyprotein beginning with the methionine residue of the C protein (nt 102-104).

Wild type amino acid on left of amino acid position; mutant amino acid on right.

**Table 11. Putative Vero cell adaptation mutations derived from the full set of 5-FU mutant viruses.**

Nucleotide position	Gene / region (a.a. #) <sup>b</sup>	5-FU mutant viruses		
		Nucleotide change	Amino acid change	No. of viruses with the mutation
1455	E (452)	G > U	Val > Phe	5
2280	E (727)	U > C	Phe > Leu	2
4891	NS3 (1597)	U > C	Ile > Thr	2
4995	NS3 (1599)	U > C	Ser > Pro	8
7153	NS4B (2351)	U > C	Val > Ala	3
7162	NS4B (2354)	U > C	Leu > Ser	4
7163	NS4B (2354)	A > U or C	Leu > Phe	7
7182	NS4B (2361)	G > A	Gly > Ser	2
7546	NS4B (2482)	C > U	Ala > Val	10
7630	NS5 (2510)	A > G	Lys > Arg	1
10275	3' UTR	A > U	n/a <sup>a</sup>	6
10279	3' UTR	A > C	n/a	4

<sup>a</sup> not applicable

<sup>b</sup> Amino acid position in DEN4 polyprotein beginning with the methionine residue of the C protein (nt 102-104) as residue #1.

**Table 12.** Mutagenic oligonucleotides used to generate recombinant DEN4 viruses containing single 5-FU mutations.

SEQ ID NO.	Recombinant virus (rDEN4-)	Nucleotide change	Amino acid change	Gene	pUC clone	RE site <sup>a</sup>	Oligonucleotide <sup>b</sup>
23	40	U > C	n/a	5' UTR	pUC- <i>Nhel</i>	<i>Bsa</i> WI	CAGTTCCAAAAcCGGAAGCTTG
24	2650	A > G	Asn > Ser	NS1	pUC-NS1	<i>Bst</i> WI	CCAAACGAGCTATcgTAacGTTCTCTGGG
25	3303	A > G	Arg > Gly	NS1	pUC-NS1	<i>Spy</i> I	GATTGTGACCCATgCGGCCCATCTTG
26	3442	A > G	Glu > Gly	NS1	pUC-NS1	<i>Bsp</i> FI	GGAGATTAGGCCgcTGAGcGgTAAAGAAAGAG
27	3540	G > A	Glu > Lys	NS2A	pUC-NS1	<i>Bsm</i> II	GTTTGTGGAAaAATGtcTGAGGAGAA
28	3575	G > A	Met > Ile	NS2A	pUC-NS1	<i>Ssp</i> I	CTAGGAAACACACATgATATTAGTTGTGG
29	3702	G > A	Ala > Thr	NS2A	pUC-NS2A	<i>Bgl</i> II	CAGATCCACCTAaCCAATaATGGCAGTG
30	3771	A > G	Arg > Gly	NS2A	pUC-NS2A	<i>Ava</i> I	GGAAACTCACcTCggGAGAGACAGC
31	4059	A > G	Ile > Val	NS2A	pUC-NS2A	<i>Bst</i> EII	TTGGGTAGAgTcAACcGCACCTCATCC
32	4062	A > G	Thr > Ala	NS2A	pUC-NS2A	<i>Bst</i> BI	GTAGAAAATAgCcGcTCCTCATCCTAG
33	4266	A > G	Ser > Gly	NS2B	pUC-NS2A	<i>Sna</i> BI	GGCGGCTTACGTaATGgGGTAGCTCAGC
34	4306	A > G	Asn > Ser	NS2B	pUC-NS2A	<i>Alu</i> NI	CTAGAGAAAGGCaGcttcGTGCAGTGG
35	4480	U > C	Val > Ala	NS2B	pUC-NS2A	<i>Msc</i> I	CCTTGGCcATTCCAGcACAATGAC
36	4812	G > A	Val > Ile	NS3	pUC-NS2A	<i>Apo</i> I	GACGTTCAaaTttTaGCCATAGAACC
37	4891	U > C	Ile > Thr	NS3	pUC-NS2A	<i>Kas</i> I	CTGGAGAAAcgGcGcGTAACATTAG
38	4896	G > U	Ala > Ser	NS3	pUC-NS2A	<i>Bst</i> EII	GAAATTGGAICgGTAACcTTAGATTTC
39	4907	A > U	Leu > Phe	NS3	pUC-NS2A	<i>Acl</i> I	GGAGCAGTAACgTttGATTTCAAACCC
40	4995	U > C	Ser > Pro	NS3	pUC-NS2A	<i>Bsa</i> II	GTTACCAAAcCIGGgGATTACGTC
41	5097	G > A	Asp > Asn	NS3	pUC-NS3	<i>Bsp</i> HI	GATTAACTATcATGaACTTACACCC
42	5695	A > G	Asp > Gly	NS3	pUC-NS3	<i>Ban</i> I	GGAAAACTTTGgcACcGAGTATCC
43	5872	C > U	Thr > Ile	NS3	pUC-NS3	<i>Bst</i> FI	TCCAGTGATaCCgGCTAGCGCTGCTC
44	6106	A > G	Glu > Gly	NS3	pUC-NS3	<i>Msc</i> I	GCCTCAGAGGtGgcCAAAAGGAAG
45	6259	U > C	Val > Ala	NS3	pUC-NS3	<i>Bgl</i> II	ACATGGAGGCaGAgATcTGGACTAGA
46	7153	U > C	Val > Ala	NS4B	pUC-NS4A	<i>Msc</i> I	AAAGCATGcCaAGGATGCTGTC
47	7162	U > C	Leu > Ser	NS4B	pUC-NS4A	<i>Bsp</i> FI	GCATAATGGACgctAAGCATGACTAAGG
48	7163	A > C	Leu > Phe	NS4B	pUC-NS4A	<i>Apa</i> LI	TTATTGCATAgTGcACgAAAAAGCATG



SEQ ID NO.	Recombinant virus (rDEN4-)	Nucleotide change	Amino acid change	Gene	pUC clone	RE site <sup>a</sup>	Oligonucleotide <sup>b</sup>
49	7174	C > U	Ala > Val	NS4B	pUC-NS4A	<i>Bsa</i> AI	GGGCCTATTATTaCgTAATGGAC
50	7182	G > A	Gly > Ser	NS4B	pUC-NS4A	n/a	CTGCAATCCTGGTgaTATTATTGC
51	7546	C > U	Ala > Val	NS4B	pUC-NS5A	<i>Acl</i> II	CTCATAAAAGAAcGttCAAACCCT
52	7630	A > G	Lys > Arg	NS5	pUC-NS5A	<i>Hga</i> I	CATTAGACAGAgcGAGTTTGAAG
53	7849	A > U	Asn > Ile	NS5	pUC-NS5A	<i>Hga</i> I	TGGCGAGcCTCAAGAtaGTGACTGAAG
54	8020	A > U	Asn > Ile	NS5	pUC-NS5A	<i>Cla</i> I	GAGTCATCaTCgataCCAACAATAG
55	8092	A > G	Glu > Gly	NS5	pUC-NS5A	<i>Eco</i> RI	CTTCAAAACCTGgcTTCTGCATCAAAG
56	8281	U > C	Leu > Ser	NS5	pUC-NS5B	<i>Xmn</i> I	CAAAGATGTTGagcAACAGGTTCAACAAC
57	8730	A > C	Asn > His	NS5	pUC-NS5B	<i>Ava</i> I	GGAAAGAAAGAAAcAcCCgAGACTGTGC
58	8872	A > G	Lys > Arg	NS5	pUC-NS5B	<i>Pvu</i> I	GGAACTGGTcGAtcgAGAAAGGGC
59	9977	G > A	Met > Ile	NS5	pUC-NS5C	<i>Sfc</i> I	CCAGTGGATaCtACaGAAAGATATGCTC
60	10186	U > C	Ile > Thr	NS5	pUC-NS5C	<i>Age</i> I	CAGGAACCTGAcCGGIAAAGAGGAATACG
61	10275	A > U	n/a	3' UTR	pUC-NS5C	n/a	CTGTAAATTACCAACATCAAAACACCAAAG
62	10279	A > C	n/a	3' UTR	pUC-NS5C	n/a	CCAACAACAAAcCACCAAAGGCTATTG
63	10634	U > C	n/a	3' UTR	pUC-3'UTR	n/a	GGATTGGTGTGTcGATCCAAACAGG

<sup>a</sup> Primers were engineered which introduced (underline) or ablated (hatched line) translationally-silent restriction enzyme sites.

<sup>b</sup> Lowercase letters indicate nt changes and bold letters indicate the site of the 5-FU mutation, which in some oligonucleotides differs from the original nucleotide substitution change in order to create a unique restriction enzyme site. The change preserves the codon for the amino acid substitution.

**TableE 13.** *sp*, *ts* and mouse attenuation phenotypes of rDEN4 mutant viruses encoding single mutations identified in six *sp* 5-FU mutant viruses.

5-FU mutant virus	Virus	Gene/region containing mutation	Mean virus titer (log <sub>10</sub> PFU/ml) at indicated temp (°C)						Replication in suckling mice <sup>b</sup>				Replication in HuH-7-SCID mice <sup>d</sup>			
			Vero cells			HuH-7 cells			n	Mean virus titer ± SE (log <sub>10</sub> PFU/g brain)	Mean log <sub>10</sub> -unit reduction from value for wt <sup>c</sup>	n	Mean peak virus titer ± SE (log <sub>10</sub> PFU/ml serum)	Mean log <sub>10</sub> -unit reduction from value for wt <sup>c</sup>		
			35	39	Δ <sup>a</sup>	35	39	Δ								
738	2A-13		7.6	7.1	0.5	7.8	6.6	1.2	30	6.5 ± 0.1	—	29	6.8 ± 0.2	—		
	rDEN4		7.6	6.8	0.8	8.0	6.7	1.3	54	5.8 ± 0.1	—	32	6.3 ± 0.2	—		
	rDEN4Δ30		7.6	6.9	0.7	7.7	5.6	2.1	30	5.6 ± 0.1	0.2	18	5.4 ± 0.2	0.9		
	parent		6.5	5.7	0.8	x6.9	3.1 <sup>e</sup>	3.8	12	4.4 ± 0.4	2.3	9	5.4 ± 0.7	1.9		
922	rDEN4-3540	NS2A	6.9	5.1	1.8	7.4	3.7	3.7	12	4.1 ± 0.3	1.7	5	6.1 ± 0.3	(+)0.1		
	rDEN4-7162	NS4B	7.2	6.8	0.4	7.4	6.6	0.8	8	5.6 ± 0.3	0.3	5	6.8 ± 0.6	0.3		
	parent		7.3	3.8	3.5	x7.4	3.0	4.4	12	3.5 ± 0.1	2.9	6	6.2 ± 0.2	0.4		
	rDEN4-4306	NS2B	x5.0	2.2	2.8	x5.6	<1.6	>4.0	12	1.7 ± 0.1	4.1	5	5.2 ± 0.6	1.1		
	rDEN4-5872	NS3	5.7	2.5	3.2	x6.5	<1.6	>4.9	12	4.5 ± 0.3	1.3	5	6.2 ± 0.5	0.1		
	rDEN4-7163	NS4B	7.8	7.2	0.6	8.0	7.4	0.6	6	6.2 ± 0.2	(+)0.1	6	5.8 ± 0.6	(+)0.2		
1081	rDEN4-3'UTR		6.9	5.7	1.2	7.7	5.7	2.0	6	4.8 ± 0.2	0.7	4	6.7 ± 0.2	0.4		
	parent		6.9	3.9	3.0	x5.8	1.9	3.9	12	2.6 ± 0.2	3.9	4	4.2 ± 0.5	2.4		
	rDEN4-2650	NS1	5.1	3.0	2.1	x5.5	2.8	2.7	12	3.0 ± 0.3	2.8	6	4.7 ± 0.5	2.2		
	rDEN4-7163	NS4B	7.8	7.2	0.6	8.0	7.4	0.6	6	6.2 ± 0.2	(+)0.1	6	5.8 ± 0.6	(+)0.2		
1083	parent		7.4	5.8	1.6	x7.4	<1.6	≥5.8	12	4.5 ± 0.4	2.0	9	4.4 ± 0.3	2.9		
	rDEN4-3702	NS2A	6.8	5.6	1.2	7.6	4.7	2.9	18	4.9 ± 0.3	0.9	7	6.3 ± 0.3	0.2		
	rDEN4-7153	NS4B	7.7	7.2	0.5	8.0	6.9	1.1	6	5.7 ± 0.1	0.2	4	5.9 ± 0.7	0.1		
	rDEN4-3'UTR		4.9	1.6	3.3	x5.7	<1.6	>4.1	12	2.4 ± 0.3	3.4	7	3.3 ± 0.4	3.6		

		Mean virus titer (log <sub>10</sub> PFU/ml) at indicated temp (°C)		Replication in suckling mice <sup>b</sup>		Replication in HuH-7-SCID mice <sup>d</sup>						
		Vero cells	HuH-7 cells									
10634												
1136	parent	5.1 <u>≤1.6</u>	≥3.5	×5.7	<u>≤1.6</u>	≥4.1	6	2.9 ± 0.3	4.5	7	4.5 ± 0.4	1.2
	rDEN4-3771 NS2A	7.0 4.6	2.4	×7.6	<u>3.7</u>	3.9	12	2.6 ± 0.4	3.2	4	6.4 ± 0.2	(+)0.1
	rDEN4-4891 NS3	7.1 <u>≤1.6</u>	>5.5	×7.4	<u>≤1.6</u>	>5.8	12	2.5 ± 0.3	3.5	6	6.0 ± 0.5	0.3
	rDEN4-3'UTR	6.9 5.8	1.1	7.1	5.2	1.9	6	5.0 ± 0.3	0.5	4	6.7 ± 0.3	0.4
10275												
1189	parent	×6.3 <u>3.8</u>	2.5	×5.5	<u>≤1.6</u>	≥3.9	12	3.2 ± 0.4	3.7	13	2.3 ± 0.3	3.8
	rDEN4-3303 NS1	6.1 4.8	1.3	6.6	3.9	2.7	8	5.7 ± 0.4	0.2	4	6.3 ± 0.3	0.8
	rDEN4-4812 NS3	7.0 6.3	0.7	7.1	6.3	0.8	12	4.8 ± 0.2	1.0	5	6.1 ± 0.5	(+)0.5
	rDEN4-5097 NS3	×5.0 <u>≤1.6</u>	>3.4	×4.6	<u>≤1.6</u>	>3.0	12	1.8 ± 0.1	4.0	8	1.9 ± 0.1	4.3
	rDEN4-7182 NS4B	7.7 6.9	0.8	7.8	6.8	1.0	6	6.2 ± 0.1	(+)0.1	6	6.3 ± 0.3	(+)0.7

<sup>a</sup> Reduction in mean virus titer ( $\log_{10}$ PFU/ml) at 39 $^{\circ}$ C compared to permissive temperature (35 $^{\circ}$ C).

<sup>b</sup> Groups of 6 suckling mice were inoculated i.c. with 10<sup>4</sup> PFU of virus. Brains were removed 5 days later, homogenized, and titered in Vero cells.

<sup>c</sup> Comparison of mean virus titers of mice inoculated with mutant virus and concurrent DEN4 control. Bold denotes  $\geq 50$ - or  $\geq 100$ -fold decrease in replication in suckling or SCID-HuH-7 mice, respectively.

<sup>d</sup> Groups of HuH-7-SCID mice were inoculated directly into the tumor with 10<sup>4</sup> PFU virus. Serum was collected on day 6 and 7 and titered in Vero cells.

<sup>e</sup> Underlined values indicate a 2.5 or 3.5  $\log_{10}$ PFU/ml reduction in titer in Vero cells or HuH-7 cells, respectively, at indicated temp when compared to permissive temp (35 $^{\circ}$ C).

<sup>\*</sup> Small plaque size at 35 $^{\circ}$ C; small plaques have a diameter of  $< 1.0$  mm compared to wild type plaque diameter of 1.5 - 2.0 mm in Vero cells, or a diameter of  $< 0.4$  mm compared to wild type plaque diameter of 0.75 to 1.0 mm in HuH-7 cells.

**Table 14. Phenotypes of rDEN4 mutant viruses encoding single mutations identified in 10 S-FU mutant viruses that are ts in both Vero and HuH-7 cells.**

S-FU mutant viruses	rDEN4-Mutation (nt position)	Gene/region	Mean virus titer (log <sub>10</sub> PFU/ml) at indicated temp (°C)										Replication in 7-day mice <sup>b</sup>		Replication in HuH-7-SCID mice <sup>d</sup>	
			Vero cells					HuH-7 cells					n	Mean log <sub>10</sub> reduction from wf (log <sub>10</sub> PFU/g brain)	n	Mean log <sub>10</sub> reduction from wf (log <sub>10</sub> PFU/ml serum)
			35	37	39	39	Δ <sup>a</sup>	35	37	38	39	Δ				
239, 489, 773	parent 4995'	NS3	7.6	6.8	5.6	3.3 <sup>a</sup>	4.3	7.6	6.7	4.7	2.5	5.1	30	2.1	6	0.3
			5.7	4.9	3.6	<1.6	>4.1	6.4	5.7	4.0	<1.6	>4.8	6	2.9		
473	parent 4480	NS2B	6.7	6.3	5.4	2.0	4.7	7.2	6.7	3.7	1.9	5.3	12	1.2	8	(+)0.3
	4995'	NS3	6.7	6.3	6.0	5.7	1.0	7.6	7.2	6.0	5.2	2.4	6	0.7		
			5.7	4.9	3.6	<1.6	>4.1	6.4	5.7	4.0	<1.6	>4.8	6	2.9		
759	parent 4995'	NS3	7.2	6.9	6.4	4.7	2.5	7.5	6.8	6.3	3.1	4.4	12	1.4	5	(+)0.4
	8020	NS5	5.7	4.9	3.6	<1.6	>4.1	6.4	5.7	4.0	<1.6	>4.8	6	2.9		
			7.1	6.6	6.7	5.9	1.2	7.4	7.1	6.1	5.4	2.0	6	0.5		
816	parent 4995'	NS3	6.8	6.4	5.8	3.9	2.9	7.5	6.2	5.5	3.1	4.4	6	2.9	6	0.4
	7174	NS4B	5.7	4.9	3.6	<1.6	>4.1	6.4	5.7	4.0	<1.6	>4.8	6	2.9		
			6.9	7.1	6.9	6.1	0.8	7.5	7.2	7.1	5.6	1.9	6	0.6		
938	parent 3442	NS1	7.1	6.5	5.6	3.1	4.0	7.2	6.4	5.6	3.1	4.1	6	1.7	6	0.5
	4995'	NS3	5.1	3.6	4.3	2.1	3.0	5.9	4.9	3.9	<1.6	4.3	6	4.1		
	10275	3'UTR	5.7	4.9	3.6	<1.6	>4.1	6.4	5.7	4.0	<1.6	>4.8	6	2.9		
			6.9	6.4	6.4	5.8	1.1	7.1	6.8	7.1	5.2	1.9	6	0.5		
173	parent 7163	NS4B	7.0	6.1	3.2	2.9	4.1	7.0	3.2	3.0	2.1	4.9	18	2.2	6	1.1
	7849	NS5	7.8	7.7	7.6	7.2	0.6	8.0	7.7	7.5	7.4	0.6	6	(+)0.1		
	8872	NS5	7.0	6.7	3.7	2.1	4.9	7.7	5.5	3.6	2.4	5.3	6	3.1		
			7.0	6.3	6.4	4.4	2.6	7.4	6.4	5.1	2.9	4.5	6	0.1		
509	parent 4266	NS2B	6.2	5.8	5.5	3.4	2.8	6.5	6.1	4.5	<1.6	>4.9	6	1.9	6	1.5
			5.9	6.1	6.1	5.2	0.7	6.7	6.1	5.7	5.3	1.4	6	1.0		

8092	NS5	5.0 <sup>a</sup>	4.6	4.6	4.6	5.6 <sup>a</sup>	4.8	4.4	4.4	4.0	12	4.0
1033	parent	6.7	6.0	5.9	4.1	2.6	6.9	5.6	4.7	1.7	12	1.7
	4907	NS3	6.7	6.0	5.8	4.0	2.7	7.1	6.1	6.8	12	1.8
	8730	NS5	7.0	6.7	6.6	6.7	0.3	7.6	7.0	7.2	12	0.6
	9977	NS5	5.6	5.5	4.6	4.1	1.5	6.4	6.1	6.2	6	0.7

<sup>a</sup> Reduction in mean virus titer ( $\log_{10}$  PFU/ml) at 39°C compared to permissive temperature (35°C).

<sup>b</sup> Groups of 6 suckling mice were inoculated i.c. with  $10^4$  PFU of virus. Brains were removed 5 days later, homogenized, and titered in Vero cells.

<sup>c</sup> Comparison of mean virus titers of mice inoculated with mutant virus and concurrent DEN4 control. Bold denotes  $\geq 50\%$  or  $\geq 100$ -fold decrease in replication in suckling or SCID-HuH-7 mice, respectively.

<sup>d</sup> Groups of HuH-7-SCID mice were inoculated directly into the tumor with  $10^4$  PFU virus. Serum was collected on day 6 and 7 and titered in Vero cells.

<sup>e</sup> Underlined values indicate a 2.5 or 3.5  $\log_{10}$  PFU/ml reduction in titer in Vero cells or HuH-7 cells, respectively, at indicated temp when compared to permissive temp (35°C).

<sup>f</sup> Data represents the results from a single rDEN4-4995 virus.

<sup>g</sup> Small plaque size at 35°C; small plaques have a diameter of  $<1.0$  mm compared to wild type plaque diameter of 1.5 - 2.0 mm in Vero cells, or a diameter of  $<0.4$  mm compared to wild type plaque diameter of 0.75 to 1.0 mm in HuH-7 cells.

**Table 15.** *sp*, *ts* and mouse attenuation phenotypes of rDEN4 mutant viruses encoding single mutations identified in 3 HuH-7 cell-specific *ts* 5-FU mutant viruses.

5-FU mutant viruses	rDEN4-Mutation (nt position)	Gene/region	Mean virus titer (log <sub>10</sub> PFU/ml) at indicated temp (°C)										Replication in 7-day mice <sup>b</sup>		Replication in HuH-7-SCID mice <sup>b</sup>	
			Vero cells					HuH-7 cells					n	Mean log <sub>10</sub> reduction from wt <sup>c</sup> (log <sub>10</sub> PFU/g brain) <sup>10</sup>	n	Mean log <sub>10</sub> reduction from wt <sup>c</sup> (log <sub>10</sub> PFU/ml serum)
			35	37	39	39	Δ <sup>a</sup>	35	37	38	39	Δ				
686	parent		7.0	6.7	6.7	6.4	0.6	7.3	6.8	6.4	2.2	5.1	12	3.8	6	1.2
	3575	NS2A	6.9	6.9	7.1	7.0	0.1	7.9	6.8	6.9	4.9	3.0	12	2.3		nd <sup>e</sup>
	4062	NS2A	6.8	6.6	6.3	4.7	2.1	6.9	6.8	7.0	<1.6	>5.3	12	2.2		nd
	7163	NS4B	7.8	7.7	7.6	7.2	0.6	8.0	7.7	7.5	7.4	0.6	6	(+)0.1		nd
992	parent		7.3	7.1	6.8	5.9	1.4	7.4	6.9	5.0	<1.6	>5.8	6	2.7	7	1.3
	5695	NS3	5.6	4.7	4.7	3.8	1.8	6.3	5.1	3.7	<1.6	>4.7	6	2.8		nd
	7162	NS4B	7.2	7.3	6.6	6.8	0.4	7.4	7.3	7.3	6.6	0.8	8	0.3		nd
1175	parent		7.4	7.1	6.9	5.3	2.1	7.6	6.5	4.7	3.3	4.3	12	1.7	5	1.0
	7153	NS4B	7.7	7.7	7.6	7.2	0.5	8.0	7.8	7.5	6.9	1.1	6	0.2		nd
	10186	NS5	4.3	3.7	2.4	<1.6	>2.7	5.1	<1.6	<1.6	<1.6	>3.5	6	3.4		nd
	10275	3'	6.9	6.4	6.4	5.8	1.1	7.1	6.8	7.1	5.2	1.9	6	0.5		nd
		UTR														

<sup>a</sup> Reduction in titer (log<sub>10</sub>PFU/ml) at 39°C compared to permissive temperature (35°C).

<sup>b</sup> Groups of 6 suckling mice were inoculated i.c. with 10<sup>4</sup> PFU virus. Brains were removed 5 days later, homogenized, and titered in Vero cells.

<sup>c</sup> Determined by comparing mean viral titers of mice inoculated with mutant virus and concurrent 2A-13 or rDEN4 wt control.

<sup>d</sup> Underlined values indicate a 2.5 or 3.5 log<sub>10</sub>PFU/ml reduction in titer in Vero cells or HuH-7 cells, respectively, at indicated temp when compared to permissive temp (35°C).

**Table 16.** Temperature-sensitive (*ts*) and mouse brain attenuation (*att*) phenotypes of additional rDEN4 viruses encoding single 5-FU mutations.

5-FU mutant virus	Gene/region containing mutation	Mean virus titer (log <sub>10</sub> PFU/ml) at indicated temp (°C)										Replication in suckling mice <sup>b</sup>	
		Vero cells					HuH-7 cells					Mean virus titer ±SE (log <sub>10</sub> PFU/g brain)	Mean log <sub>10</sub> -unit reduction from value for wt <sup>c</sup>
		35	37	38	39	Δ <sup>a</sup>	35	37	38	39	Δ		
695	rDEN4-40	7.4	7.2	6.7	6.2	1.2	7.6	7.5	7.1	5.8	1.8	nd <sup>f</sup>	nd
718	rDEN4-4059	7.0	6.7	6.4	6.2	0.8	7.7	7.1	7.0	6.6	1.1	nd	nd
311	rDEN4-4896	7.0	6.1	5.9	4.2	2.8	6.9 <sup>x</sup>	6.0	5.6	3.3	3.6	4.1 ± 0.4	2.0 <sup>**</sup>
695	rDEN4-6106	6.8	6.3	5.9	3.9	2.9	7.1	6.0	5.2	3.4	3.7	nd	nd
631	rDEN4-6259	7.0	6.1	5.8	5.0	2.0	7.5	6.6	5.7	4.2	3.3	2.2 ± 0.2	3.9 <sup>**</sup>
695 <sup>e</sup>	rDEN4-7546	7.5	7.6	7.4	6.6	0.9	7.7	7.6	7.3	5.7	2.0	nd	nd
718	rDEN4-7630	7.0	6.9	6.9	6.4	0.6	7.4	7.4	7.2	6.8	0.6	5.0 ± 0.3	0.5
718	rDEN4-8281	6.4	6.6	6.7	5.4	1.0	7.6	7.6	7.0	5.1	2.5	5.0 ± 0.5	1.1

<sup>a</sup> Reduction in titer (log<sub>10</sub>PFU/ml) at 39°C compared to titer at permissive temperature (35°C).

<sup>b</sup> 6 mice were inoculated i.c. with 10<sup>4</sup> PFU virus in 30μl inoculum. Brains were removed 5 days later, homogenized, and titered on Vero cells. Limit of detection is 2.0 log<sub>10</sub>PFU/g.

<sup>c</sup> Determined by comparing mean viral titers of mice inoculated with sample virus and wt rDEN4 control.

<sup>d</sup> Underlined values indicate a 2.5 or 3.5 log<sub>10</sub>PFU/ml reduction in titer in Vero cells or HuH-7 cells, respectively, at indicated temperature when compared to permissive temperature (35°C).

<sup>e</sup> The 7546 mutation is also present in nine other 5-FU mutant viruses.

<sup>x</sup> Small plaque size at 35°C; small plaques have a diameter of <0.4 mm compared to wt plaque diameter of 0.75 to 1.0 mm in HuH-7 cells.

<sup>f</sup> not determined

<sup>\*\*</sup> The *att* phenotype is defined as a reduction of >1.5 log<sub>10</sub>PFU/g compared to wt virus.

**Table 17. Growth of wt DEN-4 2A-13 in SCID mice transplanted with HuH-7 cells.<sup>a</sup>**

Dose (log <sub>10</sub> PFU/ml)	Mouse #	Virus titer				
		log <sub>10</sub> PFU/ml serum		log <sub>10</sub> PFU/g tissue		
		day 3	day 5	Brain	Liver	Tumor
4	87	2.7	5.9	2.0	6.9	8.0
	88	2.0	5.9	3.8	3.3	8.0
	89	<1.7	6.2	2.7	3.6	8.0
	90	1.7	3.5	3.2	3.0	7.0
5	84	<1.7	7.2	3.2	4.0	7.0
	85	1.7	6.6	3.6	6.3	5.8
6	91	4.4	8.3	6.0	7.3	8.0
	92	4.2	7.7	3.3	6.9	7.3
	93	4.0	6.6	3.3	5.7	8.4
	94	4.3	8.1	5.8	7.8	7.5

<sup>a</sup> SCID mice were injected i.p. with 10<sup>7</sup> HuH-7 human hepatoma cells. Approximately 8 weeks later, groups of tumor-bearing SCID-HuH-7 mice were inoculated with virus directly into the tumor. Serum and tissues were collected on day 5, processed, and titered in Vero cells.





**Table 19.** The 5-FU mutations are compatible with the  $\Delta 30$  mutation for replication in the brain of suckling mice.

Virus	No. of mice/ group	Mean virus titer $\pm$ SE (log <sub>10</sub> PFU/g brain) <sup>a</sup>	Mean log <sub>10</sub> -unit reduction from wt <sup>b</sup>
rDEN4	12	6.0 $\pm$ 0.1	—
rDEN4 $\Delta$ 30	12	5.3 $\pm$ 0.1	0.7
rDEN4-2650 <sup>c</sup>	12	3.7 $\pm$ 0.2	2.3
rDEN4 $\Delta$ 30-2650	12	3.9 $\pm$ 0.1	2.1
rDEN4-4995 <sup>d</sup>	6	3.5 $\pm$ 0.2	2.5
rDEN4 $\Delta$ 30-4995	6	2.7 $\pm$ 0.4	3.3
rDEN4-8092 <sup>d</sup>	12	2.0 $\pm$ 0.1	4.0
rDEN4 $\Delta$ 30-8092	6	3.2 $\pm$ 0.2	2.8
rDEN4-10634 <sup>c</sup>	12	3.8 $\pm$ 0.1	2.2
rDEN4 $\Delta$ 30-10634	12	3.6 $\pm$ 0.1	2.4

<sup>a</sup> Groups of 6 suckling mice were inoculated i.c. with 10<sup>4</sup> PFU of virus. Brains were removed 5 days later, homogenized, and titered in Vero cells.

<sup>b</sup> Comparison of mean virus titers of mice inoculated with mutant virus and rDEN4 control.

<sup>c</sup> Mutation restricts growth in both mouse brain and HuH-7-SCID mice.

<sup>d</sup> Mutation restricts growth in mouse brain only. The 8092 mutation has not been tested in SCID-HuH7 mice.

**Table 20. Temperature-sensitive and mouse brain attenuation phenotypes of viruses bearing charge-cluster-to-alanine mutations in the NS5 gene of DEN4.**

Mutation <sup>a</sup>	Changed AA Pair	# nt changed	Mean virus titer (log <sub>10</sub> PFU/ml at indicated temperature (°C)) <sup>b</sup>										Replication in suckling mice <sup>d</sup>		
			Vero Cells					HuH-7 Cells					n	Mean titer ± SE (log <sub>10</sub> PFU/g brain)	Mean log reduction from wt <sup>e</sup>
			35	37	38	39	Δ <sup>c</sup>	35	37	38	39	Δ			
wt (rDEN4)	n/a	0	8.1	8.1	7.9	7.6	0.5	8.3	8.0	7.5	7.5	0.8	48	6.0 ± 0.16	-
deletion (rDEN4Δ30)	n/a	30	6.3	6.1	6.1	5.7	0.6	6.9	6.3	5.9	4.7	2.2	42	5.4 ± 0.22	0.6
21-22	DR	4	7.2	6.8	6.7	6.1	1.1	7.6	7.1	7.0	4.7	2.9	6	5.0 ± 0.50	0.6
22-23	RK	4	7.0	7.8	6.9	3.7	3.3	7.6	7.6	6.5	<1.7	>5.9	6	2.6 ± 0.19	2.9
23-24	KE	3	6.7	6.6	6.0	6.5	0.2	7.1	7.3	5.6	<1.7	>5.4	18	4.7 ± 0.09	1.5
26-27	EE	3	7.8	7.6	6.8	4.0	3.8	8.4	8.2	7.3	4.9	3.5	6	5.7 ± 0.30	+0.1
46-47	KD	3	7.4	7.4	7.3	7.0	0.4	7.8	7.8	7.3	6.8	1.0	6	5.4 ± 0.42	0.5
157-158	EE	3	6.5	7.2	5.1	5.1	1.4	7.6	7.4	5.9	<1.7	>5.9	6	2.8 ± 0.31	2.7
200-201	KH	4	5.3	4.6	5.3	4.1	1.2	5.6	4.9	3.7	<1.7	>3.9	12	5.5 ± 0.45	0.8
246-247	RH	5	6.9	5.8	5.7	5.4	1.5	6.4	6.1	6.1	5.5	0.9	6	6.1 ± 0.17	+0.5
253-254	EK	4	7.1	6.9	6.8	7.0	0.1	7.9	7.5	7.6	6.8	1.1	6	6.2 ± 0.13	+0.6
356-357	KE	3	7.7	7.6	7.0	7.0	0.7	8.0	7.3	6.4	<1.7	>6.3	6	3.5 ± 0.58	2.0
387-388	KK	5	7.7	6.1	7.0	<1.7	>6.0	7.0	6.3	7.0	<1.7	>5.3	6	3.1 ± 0.33	2.4
388-389	KK	5	5.1	4.5	<1.7	<1.7	>3.4	6.1	5.0	<1.7	<1.7	>4.4	6	5.0 ± 0.23	1.4
396-397	RE	4	7.0	7.3	6.5	5.5	1.5	7.5	7.6	7.5	<1.7	>5.8	18	5.4 ± 0.35	1.1
397-398	EE	2	7.0	7.1	7.0	3.0	4.0	8.0	7.6	7.0	<1.7	>6.3	6	6.0 ± 0.22	0.8
436-437	DK	4	4.5	3.3	3.0	2.0	2.5	5.7	4.5	<1.7	<1.7	>4.0	12	2.3 ± 0.14	3.9
500-501	RE	3	6.6	6.3	5.7	2.3	4.3	7.1	6.5	<1.7	<1.7	>5.4	6	6.9 ± 0.49	+0.7
520-521	EE	3	5.6	4.7	4.3	<1.7	>3.9	6.7	5.7	<1.7	<1.7	>5.0	6	5.2 ± 0.48	0.2
523-524	DK	4	6.6	6.3	6.3	5.8	0.8	7.1	6.6	<1.7	<1.7	>5.4	6	4.2 ± 0.47	1.3
524-525	KK	5	7.1	6.9	6.9	6.6	0.5	7.8	7.4	7.0	5.3	2.5	6	3.4 ± 0.54	2.1
525-526	KD	4	7.8	7.1	7.6	6.8	1.0	7.9	7.7	8.0	6.9	1.0	6	3.7 ± 0.64	1.8
596-597	KD	3	4.6	4.0	2.6	<1.7	>2.9	5.7	4.9	4.0	<1.7	>4.0	6	5.9 ± 0.14	0.5
641-642	KE	4	7.3	6.9	6.9	5.2	2.1	7.8	7.5	7.2	6.9	0.9	6	4.7 ± 0.45	1.2
642-643	ER	3	6.8	6.1	4.0	3.3	3.5	7.5	7.1	6.6	3.0	4.5	12	2.6 ± 0.15	3.6
645-646	EK	4	6.3	5.3	5.9	3.1	3.2	6.4	5.8	5.5	4.5	1.9	6	5.4 ± 0.51	0.2
649-650	KE	3	6.9	6.8	6.9	6.3	0.6	7.1	7.3	7.5	7.0	0.1	12	6.4 ± 0.20	+0.2
654-655	DR	4	6.3	5.7	<1.7	<1.7	>4.6	7.0	7.1	4.6	<1.7	>5.3	12	1.8 ± 0.10	4.0

750-751	RE	3	7.1	7.1	6.9	5.7	1.4	7.8	6.9	6.5	5.6	2.2	6	6.0 ± 0.18	0.7
808-809	ED	3	4.6	4.1	<u>&lt;1.7</u>	<u>&lt;1.7</u>	>2.9	5.2	<u>&lt;1.7</u>	<u>&lt;1.7</u>	<u>&lt;1.7</u>	>3.5	6	1.8 ± 0.05	3.1
820-821	ED	2	6.3	6.3	5.6	<u>&lt;1.7</u>	>4.6	6.9	6.0	5.7	<u>&lt;1.7</u>	>5.2	6	5n5 ± 0.33	1.2
827-828	DK	4	6.9	6.3	6.3	5.9	1.0	7.5	6.9	5.0	<u>&lt;1.7</u>	>5.8	6	3.6 ± 0.76	2.3
877-878	KE	3	7.6	7.3	7.0	7.0	0.6	7.9	7.9	7.3	5.8	2.1	12	4.4 ± 0.65	1.8
878-879	EE	3	7.6	7.3	7.3	7.1	0.5	8.1	8.1	7.9	6.6	1.5	12	2.4 ± 0.10	3.8

<sup>a</sup> Positions of the amino acid pair mutated to an alanine pair; numbering starts at the amino terminus of the NS5 protein.

<sup>b</sup> Underlined values indicate a 2.5 or 3.5 log10 PFU/ml reduction in titer in Vero or HuH-7 cells, respectively, at the indicated temperatures when compared to permissive temperature (35°C).

<sup>c</sup> Reduction in titer (log10 PFU/ml) at 39°C compared to permissive temperature (35°C).

<sup>d</sup> Groups of six mice were inoculated i.c. with 4.0 log10 PFU virus in a 30 µl inoculum. The brain was removed 5 days later, homogenized, and titered in Vero cells.

<sup>e</sup> Determined by comparing mean viral titers in mice inoculated with sample virus and concurrent wt controls (n = 6). The attenuation phenotype is defined as a reduction of ≥1.5 log10 PFU/g compared to wt virus; reductions of ≥1.5 are listed in boldface.

**Table 21. SCID-HuH-7 attenuation phenotypes of viruses bearing charge-cluster-to-alanine mutations in the NS5 gene of DEN4.**

Mutation <sup>a</sup>	AA changed	Replication in SCID-HuH-7 mice <sup>b</sup>		
		n	Mean peak virus titer $\pm$ SE (log <sub>10</sub> PFU/ml serum)	Mean log reduction from wt <sup>c</sup>
<i>wt</i>	<i>na</i>	21	5.4 $\pm$ 0.4	-
<b><math>\Delta</math>30</b>	<i>na</i>	4	3.7 $\pm$ 0.6	<b>2.5</b>
23-24	KE	19	4.7 $\pm$ 0.5	1.3
157-158	EE	6	4.6 $\pm$ 0.6	1.3
200-201	KH	12	3.7 $\pm$ 0.2	<b>2.6</b>
356-357	KE	10	6.3 $\pm$ 0.7	(-) 1.1
396-397	RE	12	4.4 $\pm$ 1.3	1.2
397-398	EE	6	6.0 $\pm$ 0.5	(-) 0.1
436-437	DK	6	3.6 $\pm$ 0.2	<b>2.6</b>
500-501	RE	8	5.1 $\pm$ 0.4	1.1
523-524	DK	5	5.3 $\pm$ 0.7	0.6
750-751	RE	8	5.1 $\pm$ 0.4	1.1
808-809	ED	8	3.2 $\pm$ 0.4	<b>3.0</b>
827-828	DK	5	2.9 $\pm$ 0.2	1.6
878-879	EE	5	4.4 $\pm$ 0.7	1.5

<sup>a</sup> Positions of the amino acid pair changed to a pair of alanines; numbering starts at the amino terminus of the NS5 protein.

<sup>b</sup> Groups of SCID-HuH-7 mice were inoculated directly into the tumor with 10<sup>4</sup> PFU virus. Serum was collected on days 6 and 7 and titered in Vero cells.

<sup>c</sup> Comparison of mean virus titers of mice inoculated with mutant virus and concurrent DEN4 control. Bold denotes a  $\geq$ 100-fold decrease in replication. A (-) sign indicates an increase in replication relative to wt.

**Table 22.** Combination of paired charge-cluster-to-alanine mutations into double-pair mutant viruses.

Mutation Pair 1	Mutation Pair 2	Recovered
23-24	200-201	Yes
23-24	356-357	Yes
23-24	396-397	Yes
23-24	523-524	Yes
23-24	827-828	No
157-158	200-201	No
157-158	356-357	No
157-158	396-397	No
157-158	523-524	Yes
157-158	827-828	No
827-828	200-201	No
827-828	356-357	No
827-828	396-397	Yes
827-828	523-524	No

**Table 23.** Temperature-sensitive and mouse brain attenuation phenotypes of double charge-cluster-to-alanine mutants of the NS5 gene of rDEN4.

Mutation <sup>a</sup>	Charged	#nt	Mean virus titer (log10 PFU/ml) at indicated temperature (°C) <sup>b</sup>										Replication in suckling mice <sup>c</sup>		
			Vero Cells					HuH-7 cells					n	Mean virus titer ± SE (log10PFU/g brain)	Mean log reduction from wt <sup>e</sup>
			35	37	38	39	Δ <sup>c</sup>	35	37	38	39	Δ			
wt	n/a	0	8.1	8.1	7.9	7.6	0.5	8.3	8.0	7.5	7.5	0.8	48	6.0 ± 0.16	-
Δ30	n/a	30	6.3	6.1	6.1	5.7	0.6	6.9	6.3	5.9	4.7	2.2	42	5.4 ± 0.22	0.6
23-24	KE	3	6.7	6.6	6.0	6.5	0.2	7.1	7.3	5.6	<1.7	>5.4	18	4.7 ± 0.09	1.5
200-201	KH	4	5.3	4.6	5.3	4.1	1.2	5.6	4.9	3.7	<1.7	>3.9	12	5.5 ± 0.45	0.8
23-24; 200-201	KE, KH	7	7.1	6.5	6.6	<1.7	>5.4	7.8	7.3	<1.7	<1.7	>6.1	6	5.8 ± 0.16	0.6
23-24	KE	3	6.7	6.6	6.0	6.5	0.2	7.1	7.3	5.6	<1.7	>5.4	18	4.7 ± 0.09	1.5
356-357	KE	3	7.7	7.6	7.0	7.0	0.7	8.0	7.3	6.4	<1.7	>6.3	6	3.5 ± 0.58	2.0
23-24; 356-357	KE, KE	6													
23-24	KE	3	6.7	6.6	6.0	6.5	0.2	7.1	7.3	5.6	<1.7	>5.4	18	4.7 ± 0.09	1.5
396-397	RE	4	7.0	7.3	6.5	5.5	1.5	7.5	7.6	7.5	<1.7	>5.8	18	5.4 ± 0.35	1.1
23-24; 396-397	KE, RE	7	6.3	4.9	<1.7	<1.7	>4.6	7.1	6.0	5.6	<1.7	>5.4	6	3.7 ± 0.44	2.7
157-158	EE	3	6.5	7.2	5.1	5.1	1.4	7.6	7.4	5.9	<1.7	>5.9	6	2.8 ± 0.31	2.7
396-397	RE	4	7.0	7.3	6.5	5.5	1.5	7.5	7.6	7.5	<1.7	>5.8	18	5.4 ± 0.35	1.1
157-158; 396-397	EE, RE	7											6	2.0 ± 0.12	4.8
157-158	EE	3	6.5	7.2	5.1	5.1	1.4	7.6	7.4	5.9	<1.7	>5.9	6	2.8 ± 0.31	2.7
523-524	DK	4	6.6	6.3	6.3	5.8	0.8	7.1	6.6	<1.7	<1.7	>5.4	6	4.2 ± 0.47	1.3
157-158; 523-524	EE, DK	7	5.6	3.9	<1.7	<1.7	>3.9	6.3	4.1	<1.7	<1.7	>4.6			
396-397	RE	4	7.0	7.3	6.5	5.5	1.5	7.5	7.6	7.5	<1.7	>5.8	6	4.8 ± 0.54	1.6

827-828	D K	4	6.9	6.3	6.3	5.9	1.0	7.5	6.9	5.0	<u>&lt;1.7</u>	>5.8	6	3.6 ± 0.76	<b>2.3</b>
396-397;827-828	R E, D K	8	7.0	6.5	6.0	<u>&lt;1.7</u>	5.3	> 6.7	5.7	<u>&lt;1.7</u>	<u>&lt;1.7</u>	> 5.0	6	4.7 ± 0.10	1.2

<sup>a</sup> Positions of the amino acid pair mutated to an alanine pair; numbering starts at the amino terminus of the NS5 protein.

<sup>b</sup> Underlined values indicate a 2.5 or 3.5 log<sub>10</sub> PFU/ml reduction in titer in Vero or HuH-7 cells respectively, at the indicated temperatures when compared to permissive temperature (35°C).

<sup>c</sup> Reduction in titer (log<sub>10</sub>PFU/ml) at 39°C compared to permissive temperature (35°C).

<sup>d</sup> Groups of six suckling mice were inoculated i.c. with 4.0 log<sub>10</sub>PFU virus in a 30 µl inoculum. Brains were removed 5 days later, homogenized, and titered in Vero cells.

<sup>e</sup> Determined by comparing mean viral titers in mice inoculated with sample virus and concurrent wt controls (*n* = 6); reductions ≥ 1.5 are listed in boldface.



**Table 24. SCID-HuH-7 attenuation phenotypes of double charge-cluster-to-alanine mutants of the NS5 gene of rDEN4.**

Mutation <sup>a</sup>	Charged AA Pair	Replication in SCID-HuH-7 mice <sup>b</sup>		
		n	Mean peak virus titer $\pm$ SE (log <sub>10</sub> PFU/ml serum)	Mean log reduction from wt <sup>c</sup>
<i>wt</i>	<i>n/a</i>	21	5.4 $\pm$ 0.4	-
$\Delta 30$	<i>n/a</i>	4	3.7 $\pm$ 0.6	<b>2.5</b>
23-24	K E	19	4.7 $\pm$ 0.5	1.3
200-201	K H	12	3.7 $\pm$ 0.2	<b>2.6</b>
23-24; 200-201	K E, K H	13	3.4 $\pm$ 0.1	<b>2.9</b>
23-24	K E	19	4.7 $\pm$ 0.5	1.3
356-357	K E	10	6.3 $\pm$ 0.7	(+) 1.1
23-24; 356-357	K E, K E	4	3.6 $\pm$ 0.3	<b>2.3</b>
23-24	K E	19	4.7 $\pm$ 0.5	1.3
396-397	R E	12	4.4 $\pm$ 1.3	1.2
23-24; 396-397	K E, R E	10	3.4 $\pm$ 0.5	<b>3.3</b>
157-158	E E	6	4.6 $\pm$ 0.6	1.3
396-397	R E	12	4.4 $\pm$ 1.3	1.2
157-158; 396-397	E E, R E	6	2.2 $\pm$ 0.2	<b>3.6</b>
157-158	E E	6	4.6 $\pm$ 0.6	1.3
523-524	D K	5	5.3 $\pm$ 0.7	0.6
157-158; 523-524	E E, D K	3	5.1 $\pm$ 0.6	0.8
396-397	R E	12	4.4 $\pm$ 1.3	1.2
827-828	D K	5	2.9 $\pm$ 0.2	1.6
396-397; 827-828	R E, D K	4	4.1 $\pm$ 0.7	0.4

<sup>a</sup> Positions of the amino acid pair mutated to an alanine pair; numbering starts at the amino terminus of the NS5 protein.

<sup>b</sup> Groups of SCID-HuH-7 mice were inoculated directly into the tumor with 10<sup>4</sup> PFU of virus. Serum was collected on days 6 and 7 and titered in Vero cells.

<sup>c</sup> Comparison of mean virus titers of mice inoculated with mutant virus and concurrent DEN4 control. Bold denotes a  $\geq 100$ -fold decrease in replication. A (+) sign indicates an increase in replication relative to wt.

**Table 25.** Phenotypes (temperature sensitivity, plaque size and replication in mouse brain and SCID-HuH-7 mice) of wt DEN4 and viruses containing the  $\Delta 30$  and 7129 mutations.

Virus ID	Mutation <sup>a</sup>	Mean virus titer (log <sub>10</sub> PFU/ml) at indicated temperature (°C)						Replication in suckling mouse brain <sup>c</sup>			Replication in SCID-HuH-7 mice <sup>e</sup>			
		VERO			HUH7			n	Mean virus titer ± SE (log <sub>10</sub> PFU/g brain)	Mean log reduction from wtd	n	Mean peak virus titer ± SE (log <sub>10</sub> PFU/ml serum) <sup>f</sup>	Mean log reduction from wtd	
		35	39	Δ <sup>b</sup>	35	39	Δ							
<i>1-TD-1A</i>	wt	7.3	6.8	0.5	8	6.8	1.2	8.3	36	6.1 ± 0.21	-	21	5.4 ± 0.4	-
<i>p4Δ30</i>	Δ30	6.6	6.5	0.1	7.4	6.4	1.0	42	5.4 ± 0.22	0.6	4	3.7 ± 0.6	2.5	
5-1A1	C7129U	6.7	6.5	0.2	7.5	6	1.5	7.6*	6	6.2 ± 0.30	0.0			
rDEN4-7129-1A	C7129U	7.3	7.0	0.3	7.6	6.3	1.3	7.5*	6	7.2 ± 0.12	(-) 0.4	4	5.4 ± 0.8	(-) 0.8
rDEN4Δ30-7129	C7129U + Δ30	7.0						7.1*						

<sup>a</sup> Position and identity of the mutated nucleotides.

<sup>b</sup> Reduction in titer ( $\log_{10}$  PFU/ml) at 39 $^{\circ}\text{C}$  compared to permissive temperature (35 $^{\circ}\text{C}$ ).

<sup>c</sup> Groups of six suckling mice were inoculated i.c. with 4.0  $\log_{10}$  PFU virus in a 30  $\mu\text{l}$  inoculum. The brain was removed 5 days later, homogenized, and titered in Vero cells.

<sup>d</sup> Determined by comparing mean viral titers in mice inoculated with sample virus and concurrent wt controls (n = 6). The attenuation phenotype is defined as a  $\geq 50$ - or  $\geq 100$ -fold decrease in replication in suckling or SCID-HuH-7 mice, respectively. A (-) sign indicates an increase in replication relative to the wt control.

<sup>e</sup> Groups of SCID-HuH-7 mice were inoculated directly into the tumor with 10<sup>4</sup> PFU virus. Serum was collected on days 6 and 7 and titered in Vero cells.

<sup>f</sup> Small plaque size.

**Table 26.** The 5-fluorouracil 5-1A1 small plaque mutant demonstrates a restriction of midgut infection following oral infection of *Aedes aegypti* mosquitoes.

Virus tested	Dose ingested (log <sub>10</sub> PFU) <sup>a</sup>	No. mosquitoes tested	Midgut-only infection <sup>b</sup>	Disseminated infection <sup>c</sup>	Total no. infected <sup>d,e</sup>
wtDEN4 (2A-13)	4.5	19	1 (5%)	17 (89%)	18 (95%)
	3.5	26	9 (35%)	7 (27%)	16 (62%)
	2.5	28	1 (4%)	0	1 (4%)
				<b>OID<sub>50</sub> = 3.9</b>	<b>OID<sub>50</sub> = 3.3</b>
5-1A1	3.5	34	4 (12%)	2 (6%)	6 (18%)
	2.5	9	0	1 (11%)	1 (11%)
	1.5	23	0	0	0
				<b>OID<sub>50</sub> ≥ 3.9</b>	

<sup>a</sup> Amount of virus ingested, assuming a 2 µl bloodmeal.

<sup>b</sup> Number (percentage) of mosquitoes with detectable dengue virus antigen in midgut tissue, but no detectable dengue virus antigen in head; mosquitoes were assayed 21 days post-feed, and dengue virus antigen was identified by IFA.

<sup>c</sup> Number (percentage) of mosquitoes with detectable dengue virus antigen in both midgut and head tissue.

<sup>d</sup> Total number (percentage) of mosquitoes with detectable dengue virus antigen.

<sup>e</sup> The proportion of total infections caused by wild type DEN4 was significantly higher than the proportion caused by 5-1A1 (logistic regression, N = 426, P < 0.0001). There were too few disseminated infection caused by 5-1A1 to permit statistical analysis.

**Table 27.** The 5-fluorouracil 5-1A1 small plaque mutant demonstrates a restriction of infection following intrathoracic inoculation of *Toxorhynchites splendens* mosquitoes.

Virus tested	Dose ingested (log <sub>10</sub> PFU) <sup>a</sup>	No. mosquitoes tested	No (%) infected <sup>c</sup>
wtDEN4 (2A-13)	4.0	5	5 (100)
	3.0	4	4 (100)
	2.0	4	1 (25)
	<b>MID<sub>50</sub> = 2.3 log<sub>10</sub>PFU</b>		
5-1A1	3.0	9	0
	2.0	7	1 (14)
	1.0	7	0
<b>MID<sub>50</sub> &gt; 3.0 log<sub>10</sub>PFU</b>			

<sup>a</sup> Amount of virus inoculated in a 0.22 µl inoculum.

<sup>b</sup> Number (percentage) of mosquitoes with detectable dengue virus antigen in head tissue; mosquitoes were assayed 14 days post-inoculation, and dengue virus antigen was identified by IFA.

<sup>c</sup> The proportion of infections caused by wild type DEN4 was significantly higher than the proportion caused by 5-1A1 (logistic regression, N = 36, P < 0.01).

**Table 28. Mutagenesis primers for the deletion or swap of sequences in DEN4 showing conserved differences from tick-borne flaviviruses.**

DEN4 nucleotides <sup>1</sup>	Type of mutation <sup>2</sup>	Mutagenesis Primer <sup>3</sup>	SEQ ID NO
10508-10530	Δ	CTGGTGGAAGCCCAACACAAAAAC	64
10508-10530	swap	CTGGTGGAAGGAAGAGAGAAATTGGCAACTCCCCAACACAAAAAC	65
10535-10544	Δ	AGACCCCCCAAGCATATTGAC	66
10535-10544	swap	AGACCCCCCAATATTCTCCTCTATAGCATATTGAC	67
10541-10544	Δ	CCCAACACAAAGCATATTGAC	68

<sup>1</sup> Nucleotides numbered 5' to 3', in the opposite direction from Figure 5.3

<sup>2</sup> Δ: deletion of specified DEN4 nucleotides; swap: exchange of specified DEN4 nucleotides with homologous sequence from Langat

<sup>3</sup> no swap mutation was made for nucleotides 10541-10544

**Table 29.** Virus titer and plaque size of 3' UTR mutant viruses in Vero and C6/36 cells.

Virus	Vero		C6/36	
	Titer (log <sub>10</sub> PFU/ml)	Plaque size <sup>1</sup>	Titer (log <sub>10</sub> PFU/ml)	Plaque size
rDEN4Δ10508-10530	8.1	wt	7.5	wt
rDEN4swap10508-10530	5.4	sp	6.6	wt
rDEN4Δ10535-10544	5.8	wt	7.0	sp
rDEN4swap10535-10544	7.0	wt	7.3	wt
rDEN4Δ10541-10544	6.4	wt	>7.0	wt

<sup>1</sup> Plaque size is designated as equivalent to wild type (wt) or ≤50% of wild type (sp) on the designated cell type.

**Table 30.** Infectivity of wt DEN4 and 3' UTR mutants for *Toxorhynchites splendens* via intrathoracic inoculation.

Virus	Dose (log <sub>10</sub> PFU) <sup>a</sup>	No. mosquitoes tested	% Infected <sup>b</sup>	MID <sub>50</sub> (log <sub>10</sub> PFU)
rDEN4 wt	3.3	6	83	2.3
	2.3	7	57	
	1.3	6	0	
	0.3	6	0	
rDEN4Δ10508-10530	4.4	8	0	
	3.4	9	11	
	2.4	4	0	

<sup>a</sup> Amount of virus inoculated in a 0.22 µl inoculum.

<sup>b</sup> Percentage of mosquitoes with detectable dengue virus antigen in head tissue; mosquitoes were assayed 14 days post-inoculation, and dengue virus antigen was identified by IFA

**Table 31. Infectivity of 3' UTR swap mutant viruses for *Aedes aegypti* fed on an infectious bloodmeal.**

Virus Tested	Dose ingested (log <sub>10</sub> PFU) <sup>a</sup>	No. Mosquitoes Tested	Total No. Infected <sup>b, c</sup>	Disseminated Infections <sup>c, d</sup>
rDEN4	3.8	18	11 (61%)	4 (22%)
	2.8	15	5 (33%)	1 (6%)
	1.8	15	0	0
			<b>OID<sub>50</sub> = 3.4</b>	<b>OID<sub>50</sub> = ≥ 4.2</b>
rDEN4swap	3.8	25	5 (20%)	2 (8%)
10535-10544	2.8	25	0	0
	1.8	20	0	0
			<b>OID<sub>50</sub> = ≥ 4.2</b>	

<sup>a</sup> Amount of virus ingested, assuming a 2 µl bloodmeal.

<sup>b</sup> Number (%) of mosquitoes with detectable dengue virus antigen in the midgut tissue; mosquitoes were assayed either 14 d post-feed and dengue virus antigen was identified by IFA.

<sup>c</sup> At a dose of 3.8 log<sub>10</sub>PFU, rDEN4swap10535-10544 infected significantly fewer mosquitoes at the midgut than wt rDEN4 (Fisher's exact test, N = 43, P< 0.01), although disseminated infections were not significantly different (Fisher's exact test, N = 43, P=0.38).

<sup>d</sup> Number (%) of mosquitoes with detectable dengue virus antigen in the head tissue.

**Table 32. Putative Vero cell adaptation mutations derived from the set of 5-FU mutant viruses and other DEN4 viruses passaged in Vero cells.**

Nucleotide position	Gene / region (a.a. #) <sup>b</sup>	5-FU mutant viruses			Other DEN viruses passaged in Vero cells		
		Nucleotide change	Amino acid change	No. of viruses with the mutation	Virus	Nucleotide change	Amino acid change
1455	E (452)	G > U	val > phe	5			
2280 <sup>1,2,3</sup>	E (727)	U > C	phe > leu	2			
4891 <sup>2,3</sup>	NS3 (1597)	U > C	ile > thr	2			
4995 <sup>1,2</sup>	NS3 (1599)	U > C	ser > pro	8			
7153	NS4B (2351)	U > C	val > ala	3	2AΔ30	U > C	val > ala
7162	NS4B (2354)	U > C	leu > ser	4	2A-1	U > C	leu > ser
7163	NS4B (2354)	A > U or C	leu > phe	7	rDEN4Δ30	A > U	leu > phe
					2A-13-1A1	A > U	leu > phe
7182 <sup>1,2,3</sup>	NS4B (2361)	G > A	gly > ser	2			
7546	NS4B (2482)	C > U	ala > val	10			
7630 <sup>3</sup>	NS5 (2510)	A > G	lys > arg	1	814669	A > G	lys > arg
10275	3' UTR	A > U	n/a <sup>c</sup>	6			
10279	3' UTR	A > C	n/a	4			

<sup>a</sup> Conservation with DEN1, DEN2, or DEN3 is designated by superscript. Lack of conservation is designated by no superscript.

<sup>b</sup> Amino acid position in DEN4 polypeptide beginning with the methionine residue of the C protein (nt 102-104) as residue #1.

<sup>c</sup> not applicable



**Table 33.** Sequence analysis of rDEN2/4Δ30 clone 27(p4)-2-2A2.

Nucleotide	Gene	Mutation	
		Nucleotide	Amino acid
743	M anchor	G > A	Gly > Glu
1493	E	C > U	Ser > Phe
7544*	NS4B	C > U	Ala > Val

\* Same as DEN4 nucleotide position 7546

**Table 34.** Sequence analysis of rDEN2/4Δ30 clone 27(p3)-2-1A1.

Nucleotide	Gene	Mutation	
		Nucleotide	Amino acid
1345	E	U > C	Tyr > His
4885*	NS3	G > A	Glu > Lys
8297	NS5	G > A	Arg > Lys

\*Codon adjacent to 5-FU mutation 4891

**Table 35.** Recombinant virus rDEN2/4Δ30 bearing Vero adaptation mutations can be recovery and titered on Vero cells.

Virus	Virus titer in indicated cell line <sup>1</sup> (log <sub>10</sub> PFU/ml)		Virus titer following recovery in Vero cells (log <sub>10</sub> PFU/ml)
	C6/36	Vero	
rDEN2/4Δ30 wt	5.2	1.7	<0.7
rDEN2/4Δ30-7153	5.4	5.2	<0.7
rDEN2/4Δ30-7162	5.4	5.3	nd <sup>2</sup>
rDEN2/4Δ30-7182	4.7	4.9	2.3
rDEN2/4Δ30-7630	5.3	4.8	1.3
rDEN2/4Δ30-7153-7163	5.1	4.7	nd
rDEN2/4Δ30-7153-7182	4.1	3.2	nd
rDEN2/4Δ30-7546-7630	5.2	5.2	nd

<sup>1</sup> Virus recovered following transfection of C6/36 mosquito cells was terminally diluted once in C6/36 cells and titered simultaneously in C6/36 cells and Vero cells.

<sup>2</sup> not determined

**Table 36.** Putative Vero cell adaptation mutations of dengue type 4 virus and the corresponding wildtype amino acid residue in other dengue viruses.

Mutation	Amino acid position <sup>a</sup>	Mutant residue	Amino acid in indicated wt dengue virus <sup>b</sup>			
			DEN4	DEN1	DEN2	DEN3
1455	452	F	V	I	A	A
2280	727	L	<u>F</u> <sup>c</sup>	<u>F</u>	<u>F</u>	<u>F</u>
4891	1597	T	<u>I</u>	V	<u>I</u>	<u>I</u>
4995	1632	P	<u>S</u>	<u>S</u>	<u>S</u>	N
7129	2343	L	<u>P</u>	<u>P</u>	<u>P</u>	<u>P</u>
7153	2351	A	V	F	F	L
7162	2354	S	L	V	V	V
7163	2354	F	L	V	V	V
7182	2361	S	<u>G</u>	<u>G</u>	<u>G</u>	<u>G</u>
7546	2482	V	A	L	T	V
7630	2510	R	K	S	S	K

<sup>a</sup> Amino acid position is given for the polyprotein of DEN4

<sup>b</sup> DEN4 = rDEN4 (GenBank AF326825); DEN1 = Western pacific (GenBank DVU88535); DEN2 = New Guinea C (GenBank AF038403); DEN3 = H87 (GenBank M93130)

<sup>c</sup> Underlined nucleotides are shared between DEN4 and one or more additional DEN types.

**Table 37. Mutations known to attenuate dengue type 4 virus and the corresponding wildtype amino acid residue in other dengue virus.**

	Mutation	Amino acid position <sup>a</sup>	Mutant residue	Amino acid in indicated wt dengue virus <sup>b</sup>			
				DEN4	DEN1	DEN2	DEN3
5-FU mutations	2650	850	S	<u>N</u> <sup>d</sup>	<u>N</u>	<u>N</u>	<u>N</u>
	3442	1114	G	<u>E</u>	<u>E</u>	<u>E</u>	<u>E</u>
	3540	1147	K	<u>E</u>	<u>E</u>	<u>E</u>	<u>E</u>
	3575	1158	I	<u>M</u>	L	A	<u>M</u>
	3771	1224	G	<u>R</u>	<u>R</u>	K	<u>R</u>
	4062	1321	A	<u>T</u>	L	A	<u>T</u>
	4306	1402	S	N	E	D	D
	4891	1597	T	<u>I</u>	V	<u>I</u>	<u>I</u>
	4896	1599	S	<u>A</u>	<u>A</u>	<u>A</u>	<u>A</u>
	4907	1602	F	<u>L</u>	<u>L</u>	<u>L</u>	<u>L</u>
	4995	1632	P	<u>S</u>	<u>S</u>	<u>S</u>	N
	5097	1666	N	<u>D</u>	<u>D</u>	<u>D</u>	<u>D</u>
	5695	1865	G	<u>D</u>	<u>D</u>	<u>D</u>	<u>D</u>
	6259	2053	A	<u>V</u>	<u>V</u>	<u>V</u>	<u>V</u>
	7129 <sup>c</sup>	2343	L	<u>P</u>	<u>P</u>	<u>P</u>	<u>P</u>
	7849	2583	I	<u>N</u>	K	<u>N</u>	K
	8092	2664	G	E	Q	Q	Q
	10186	3362	T	<u>I</u>	<u>I</u>	<u>I</u>	<u>I</u>
	10634	3' UTR	-	-	-	-	-
Charge-cluster-to-alanine mutations	22, 23	2509, 2510	AA	<u>RK</u>	KS	KS	<u>RK</u>
	23, 24	2510, 2511	AA	<u>KE</u>	SE	SE	<u>KE</u>
	157, 158	2644, 2645	AA	<u>EE</u>	<u>EE</u>	EA	<u>EE</u>
	200, 201	2687, 2688	AA	<u>KH</u>	<u>KH</u>	KY	<u>KH</u>
	356, 357	2843, 2844	AA	<u>KE</u>	<u>KE</u>	<u>KE</u>	<u>KE</u>
	387, 388	2874, 2875	AA	<u>KK</u>	RN	<u>KK</u>	RN
	436, 437	2923, 2924	AA	<u>DK</u>	HR	<u>DK</u>	<u>DK</u>
	524, 525	3011, 3012	AA	<u>KK</u>	KI	<u>KK</u>	KI
	525, 526	3012, 3013	AA	KD	IP	KE	IP
	642, 643	3129, 3130	AA	<u>ER</u>	<u>ER</u>	IA	KK
	654, 655	3141, 3142	AA	DR	ER	ER	ER
	808, 809	3295, 3296	AA	<u>ED</u>	<u>ED</u>	<u>ED</u>	<u>ED</u>
	827, 828	3314, 3315	AA	<u>DK</u>	<u>DK</u>	<u>DK</u>	<u>DK</u>
	877, 878	3364, 3365	AA	KE	NE	NE	NE
	878, 879	3365, 3366	AA	<u>EE</u>	EN	<u>EE</u>	<u>EE</u>

<sup>a</sup> Amino acid position is given for the polyprotein of DEN4

<sup>b</sup> DEN4 = rDEN4 (GenBank AF326825); DEN1 = Western pacific (GenBank U88535); DEN2 = New Guinea C (GenBank AF038403); DEN3 = H87 (GenBank M93130)

- <sup>c</sup> This mutation results in decreased replication of DEN4 in mosquitoes.
- <sup>d</sup> Underlined nucleotides are shared between DEN4 and one or more additional DEN types.